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Factor H-Binding Protein Is Important for Meningococcal Survival in Human Whole Blood and Serum and in the Presence of the Antimicrobial Peptide LL-37[▽]

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Factor H-binding protein (fHBP; GNA1870) is one of the antigens of the recombinant vaccine against serogroup B *Neisseria meningitidis*, which has been developed using reverse vaccinology and is the basis of a meningococcal B vaccine entering phase III clinical trials. Binding of factor H (fH), an inhibitor of the complement alternative pathway, to fHBP enables *N. meningitidis* to evade killing by the innate immune system. All fHBP null mutant strains analyzed were sensitive to killing in ex vivo human whole blood and serum models of meningococcal bacteremia with respect to the isogenic wild-type strains. The fHBP mutant strains of MC58 and BZ83 (high fHBP expressors) survived in human blood and serum for less than 60 min (decrease of >2 $\log_{10}$ CFU), while NZ98/254 (intermediate fHBP expressor) and 67/00 (low fHBP expressor) showed decreases of >1 $\log_{10}$ CFU after 60 to 120 min of incubation. In addition, fHBP is important for survival in the presence of the antimicrobial peptide LL-37 (decrease of >3 $\log_{10}$ CFU after 2 h of incubation), most likely due to electrostatic interactions between fHBP and the cationic LL-37 molecule. Hence, the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum and in the presence of LL-37, even at low levels. The functional significance of fHBP in mediating resistance to the human immune response, in addition to its widespread distribution and its ability to induce bactericidal antibodies, indicates that it is an important component of the serogroup B meningococcal vaccine.

Disease caused by *Neisseria meningitidis* remains a major worldwide cause of morbidity and mortality, even after the development of vaccines to protect against several meningococcal serogroups. *N. meningitidis* colonizes the mucosa of the nasopharynx in 5 to 10% of the population, and in susceptible individuals the bacterium can cross the epithelial layer into the bloodstream, causing septicemia and/or meningitis (36). The development of meningococcal serogroup A, C, W-135, and Y capsular polysaccharide-based vaccines, as well as tailor-made meningococcal serogroup B outer membrane vesicle (OMV) vaccines, has dramatically reduced the incidence of disease in areas where these vaccines have been widely used (reviewed in reference 40). Serogroup B (MenB) meningococcal strains cause one-third of meningococcal disease in the United States and up to 80% of cases in Europe, yet there is no comprehensive vaccine available to protect against this serogroup. However, the in silico genome-based approach, termed reverse vaccinology, has led to the development of a recombinant 5-*c*omponent *v*accine against *m*eningococcal serogroup *B* strains (5CVMB), which forms the basis of a MenB vaccine entering phase III clinical trials. Initial results indicate that this vaccine is well tolerated and induces bactericidal antibodies against several genetically diverse serogroup B *N. meningitidis* strains (10, 33). The MenB vaccine contains the genome-derived *Neisseria* antigens GNA1870 (fHBP), GNA1994 (NadA), GNA2132, GNA1030, and GNA2091 (10).

GNA1870 is a 28-kDa surface-exposed lipoprotein that binds factor H (fH), a key inhibitor of the complement alternative pathway (AP), leading to evasion of killing by the innate immune system. GNA1870 has thus been named fH-binding protein (fHBP) (25). This antigen is expressed by all *N. meningitidis* strains studied to date, but the level of expression varies between strains (with high, intermediate, and low expressors). fHBP has also been found in the culture supernatant of strain MC58 (26). fHBP induces high levels of bactericidal antibodies, and even the very low expressors are efficiently killed in serum bactericidal activity (SBA) assays (44), which are used as the main correlate of protection for meningococcal disease (42). In addition, anti-fHBP antibodies can block binding of fH, increasing the susceptibility to killing by the complement AP (2, 25). However, it was recently suggested that unlike fHBP of high expressors, fHBP of low expressors is not required for survival in nonimmune human blood or in the presence of certain anti-fHBP monoclonal antibodies (MAbs) (43). Antibodies to fHBP (MAbs [44] and polyclonal antibodies [26] to the recombinant protein or antibodies elicited by an fHBP-enriched OMV vaccine [16]) also confer passive protection against *N. meningitidis* bacteremia in an infant rat model.

fHBP can be classified into three allelic groups (variants 1, 2, and 3) based on its amino acid sequence. Conservation within the allelic groups ranges from 91.6% to 100%, while conservation between these groups is as low as 62% (19, 26). fH can bind to all three fHBP variants (25), but bactericidal antibodies induced by fHBP are variant specific. Variant 1 fHBP is found in approximately 60% of disease-producing serogroup B isolates (44) and is present in the 5CVMB MenB vaccine (10). fHBP, called LP2086, is the principal antigen of another MenB

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vaccine, currently in clinical trials, containing recombinant proteins from two different variant subfamilies (reviewed in references 3 and 45). Additional vaccines being investigated are focused on OMVs isolated from a strain overexpressing variant 1 fHBP (16, 21) as well as on recombinant chimeric fHBP (1).

Some nonbactericidal anti-fHBP antibodies are able to confer passive protection against meningococcal infection in the infant rat animal model, most likely via opsonophagocytosis (OP) (16) or interactions with other components of the immune response. LL-37, a cationic antimicrobial peptide, is a key component of the human innate immune system that is constitutively expressed by leukocytes, including monocytes, neutrophils, T cells, NK cells, and B cells, and inducibly expressed by epithelial cells (7), including the epithelial cells of the nasopharynx (24). LL-37 is a short, positively charged, α-helical peptide that has a broad spectrum of antimicrobial activity, through its direct targeting and disruption of the negatively charged bacterial surface membrane (31) as well as its immunoregulatory roles (4). The MtrCDE efflux pump, lipid A modification, the type IV pilin secretion system, and the capsule (39, 41) are all involved in protection of *N. meningitidis* against killing by antimicrobial peptides. Two hypothetical genes of *N. meningitidis*, NMB0741 and NMB1828, were recently shown to be upregulated in the presence of antimicrobial peptide (along with 19 other genes that are differentially expressed in the presence of CRAMP) and to play a role in cathelicidin resistance (9).

To better characterize the functional role of fHBP, the survival of four strains of *N. meningitidis* shown to express different levels of fHBP variant 1 (26) and of their fHBP mutant derivative strains was investigated in ex vivo human whole blood and serum models of meningococcal bacteremia, as well as in survival assays performed in vitro in the presence of antimicrobial peptides (LL-37 and polymyxin B) and other environmental stresses. The results presented show that the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum, regardless of the level of expression. We also show that fHBP increases meningococcal survival in the presence of the antimicrobial peptide LL-37, which is most likely mediated by electrostatic interactions between fHBP and LL-37 at the cell surface.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. meningitidis* strains used in this study are described in Table 1. *N. meningitidis* strains were routinely grown on GC agar (Difco) or Mueller-Hinton (MH) agar (Difco) at 37°C and 5% CO_2 overnight. For liquid cultures, overnight growth was used to inoculate GC or MH broth. *Escherichia coli* strains used for cloning were cultured in Luria-Bertani (LB) broth or on LB agar (Difco). When required, erythromycin and/or chloramphenicol was added to achieve a final concentration of 5 μg/ml.

Recombinant DNA techniques. Recombinant DNA techniques were routinely performed as described by Sambrook et al. (37). Plasmid DNA preparations and purification of DNA fragments from PCR samples and agarose were performed using Qiagen kits according to the manufacturer's instructions. PCR amplification was carried out in 50-μl reaction mixtures consisting of 1$\times$ reaction buffer, 1.5 mM $MgCl_2$, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1 unit of Platinum *Taq* polymerase (Invitrogen), 10 pmol each of forward and reverse primers, an appropriate amount of chromosomal or plasmid DNA, and sterilized deionized water. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA).

Construction of fHBP knockout *N. meningitidis* strains. The isogenic MC58 wild type and MC58 ΔfHBP (previously called MC58 Δgna1870) knockout mutant, in which the gna1870 gene was truncated and replaced with an erythromycin antibiotic resistance cassette, have been described previously (26). The pBSΔ*gna1870*ERM knockout plasmid was used to generate isogenic knockout mutants in BZ83, NZ98/254, and 67/00, namely, BZ83 ΔfHBP, NZ98/254 ΔfHBP, and 67/00 ΔfHBP, as described previously (26). Complementation of the MC58 ΔfHBP null mutant was achieved by insertion of the gna1870 gene under the control of the P_{tac} promoter, along with the chloramphenicol resistance gene, into a noncoding chromosomal location between the two converging open reading frames NMB1428 and NMB1429 through transformation of the MC58 ΔfHBP strain with the pComfHBP complementation plasmid, generated as follows. The P_{tac} promoter and ribosome binding site were amplified using the Tac1 (ATTCGGGTACCGGCGCACTCCCGTTCTGGATA) and Pind-R (AATGCATGCATGGTCATATGTGTTTCCTGTGAATTG) primers and cloned as a 234-bp KpnI-NsiI fragment adjacent to the chloramphenicol resistance cassette into the pSLComCmr plasmid (17), generating $pComP_{RBS}$. The gna1870 gene was amplified with primers 741-F2 (GGAATTC<u>CATATG</u>GTGAATCGAACTGCCTTC) and 741-R2 (CCA<u>ATGCAT</u>TTATTGCTTGGCGGCAAG) from the MC58 genome and cloned as a 760-bp NdeI-NsiI fragment into the $pComP_{RBS}$ plasmid, generating pComfHBP. Transformants were selected on chloramphenicol and checked by PCR, and complementation of the mutant strain was verified by Western blot analysis. The resulting complemented strain was named MC58 ΔfHBP_C.

Western blot analysis. Expression of fHBP in whole-cell samples and culture supernatants was performed essentially as described previously (26). Briefly, strains were grown at 37°C and 5% CO_2 in MH broth plus 0.25% glucose from an optical density at 600 nm (OD_{600}) of 0.05 to an OD_{600} of 0.5. Bacterial cells were collected by centrifugation, washed once with phosphate-buffered saline (PBS), and resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer. Culture supernatant was filtered using a 0.2-μm filter, and 1 ml was precipitated by the addition of 250 μl of 50% trichloroacetic acid. The sample was incubated on ice for 2 h and centrifuged for 40 min at 4°C, and the pellet was washed with 70% ice-cold ethanol and resuspended in PBS. Samples were run in 4 to 12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes. Ponceau staining was performed to ensure equal loading of the samples. Western blot analysis was performed according to standard procedures, using anti-fHBP polyclonal antibodies at a 1:1,000 dilution (raised in mice against purified recombinant fHBP [rfHBP]), as described previously (26), followed by a 1:5,000 dilution of horseradish peroxidase-labeled anti-mouse immunoglobulin G (Sigma-Aldrich).

Ex vivo human whole blood and human serum models of meningococcal bacteremia. Cells were harvested into MH broth containing 0.25% glucose and 0.02 mM CMP–*N*-acetylneuraminic acid sodium salt to an OD_{600} of 0.05, grown to mid-log phase (OD_{600}, 0.5 to 0.6), and then diluted in MH broth to approximately 10^3 CFU/ml. The assay was started by the addition of 240 μl whole human blood, human serum, or heat-inactivated human serum (56°C at 30 min) to 10 μl of bacterial suspension. Cultures were incubated at 37°C and 5% CO_2 with gentle agitation, and at various time points an aliquot of the sample was removed and the number of viable CFU were determined by plating serial dilutions onto MH agar and incubating them overnight at 37°C and 5% CO_2. Experiments were performed in triplicate on several occasions. Student's *t* test was used to determine the statistical significance of survival of each mutant strain with respect to that of the isogenic wild-type strain, with P values of <0.05 considered significant.

Whole venous blood, collected from healthy individuals (not immunized against *N. meningitidis*) and anticoagulated with heparin (10 U/ml), was used for whole-blood experiments as described by Ison et al. (18). For preparation of human serum, whole blood was coagulated at 25°C for 30 min and centrifuged at 1,000 $\times$ *g* for 10 min at 4°C, and the supernatant was retained.

In vitro antimicrobial peptide and environmental stress assays. Overnight cultures were harvested into GC broth and diluted to approximately 10^4 CFU/ml. Each assay was started by the addition of 10 μl of a compound to 90 μl of the bacterial suspension. Compounds used were as follows: LL-37 (final concentration, 2 to 7 μM; Innovagen, Sweden), polymyxin B (5 to 50 μg), detergent (0.01 to 0.1% SDS, Tween, or Triton X), reactive oxygen species (2 to 10 mM H_2O_2, 0.005 to 0.02% cumene hydroperoxide), and generators of reactive oxygen species (2.5 to 10 mM paraquat, 1 mM xanthine, and 200 to 400 mU xanthine oxidase). For osmotic stress assays, 10 μl of bacterial suspension was added to 90 μl of either NaCl (2 to 5 M) or sucrose (10 to 30%) solution. Cultures were incubated at 37°C and 5% CO_2 with gentle agitation, and CFU were determined as described above.

For some experiments, GC broth with a pH of 5, 6, 7, 8, or 9 or 2% NaCl was used to investigate the influence of pH and NaCl concentration on LL-37-mediated killing. In some cases, LL-37 was also incubated in PBS with various

TABLE 1. Summary of wild-type *Neisseria meningitidis* strains used in this study

Strain	Country of origin (yr)	Serological classification	ET cluster (ST)	fHBP variant[a] (% ID)[b]	fHBP expression	Reference
MC58	United Kingdom (1985)	B:15:P1.7, 16	ET5 (ST-74)	1.1 (100)	High	6
BZ83	The Netherlands (1984)	B:15:P−	ET5 (ST-32)	1.1 (100)	High	6
NZ98/254	New Zealand (1998)	B:4:P1.4	Lineage 3 (ST-42)	1.10 (92)	Medium	6
67/00	Norway (2000)	B:P1	Lineage 3 (ST-41/44)	1.10 (92)	Low	6

[a] fHBP variant nomenclature is described in reference 21.
[b] % identity to fHBP of MC58.

concentrations of rfHBP or cell extract for 15 min prior to the assay, as described below.

Growth in the presence of LL-37 was monitored using the MC58 wild-type, MC58 ΔfHBP, and MC58 ΔfHBP_C strains inoculated into GC broth to an OD_{600} of 0.05. Bacteria were incubated at 37°C and 5% CO_2, with or without 1 μM LL-37, and growth was followed for 5 h by reading the OD_{600}.

Investigation of LL-37–fHBP interactions by peptidase activity assay. The effect of fHBP on LL-37 was investigated using either rfHBP (26) or cell extracts from strains MC58 and MC58 ΔfHBP. Cell extracts of overnight cultures of *N. meningitidis* were prepared by resuspending the cells in PBS, followed by three cycles of freezing and thawing. Unbroken cells and cell debris were removed by centrifugation at 13,000 rpm for 10 min, and the supernatant was collected and passed through 0.2-μm filters. LL-37 (10 μg) was incubated with rfHBP (10 μg) or 10 μl of cell extract in PBS at 37°C, samples were taken at 1 min, 5 min, 15 min, 1 h, and 15 h, and the reactions were stopped by boiling for 5 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (4 to 12% Tris-Tricine gel) and stained with 0.25% Coomassie brilliant blue.

Far-Western dot blot. Five-microliter spots containing 1.25 or 2.5 μg of bovine serum albumin, rfHBP, or LL-37 or 5 μl of bacterial suspension in PBS (OD_{600} of 1) was applied to a nitrocellulose filter. The filter was air dried for 15 min, blocked in 10% milk powder in PBS-0.05% Tween (PBST) overnight, and then incubated with 4.5 μg LL-37 in 8 ml PBS for 1 h at room temperature. The membrane was washed three times in PBST and then treated as for a normal Western blot, with anti-LL-37 polyclonal antibody (Innovagen) added at a 1:1,000 dilution, followed by a 1:5,000 dilution of horseradish peroxidase-labeled anti-rabbit immunoglobulin G (Sigma-Aldrich).

Size-exclusion gel chromatography. Purified recombinant fHBP (98 μg) (26) was incubated with LL-37 (156 μg [1:10 molar ratio] or 78 μg [1:5 molar ratio]) in PBS for 1 h and then loaded onto a High-Load Superdex_75_10/300 gel filtration column (GE Healthcare) equilibrated with PBS, and 0.5-ml fractions were eluted at a flow rate of 0.8 ml/min. Fractions were analyzed by Western blotting using anti-fHBP and anti-LL-37 polyclonal antibodies as described above.

RESULTS

Analysis of fHBP expression and localization in various *Neisseria* strains. Four strains of *N. meningitidis* shown to express different levels of fHBP variant 1 (26) were used in this study to investigate the role of meningococcal fHBP in response to various components of the human immune system (see Table 1 for strain details). Western blot analysis of whole-cell extracts of these strains confirmed the different levels of fHBP expression (MC58 and BZ83 [high expressors] > NZ98/254 [intermediate expressor] > 67/00 [low expressor]) (Fig. 1A). The culture supernatants of these strains were also investigated, and like the case for MC58 (26), fHBP was present in the culture supernatants of all strains studied (Fig. 1B). No fHBP was detected in the whole-cell extract or the supernatant for the isogenic fHBP knockout mutant strains. Furthermore, fHBP was once again expressed in the complemented strain MC58 ΔfHBP_C, in which the gene encoding fHBP was inserted in *trans* under the control of the P_{tac} promoter, but expression was not fully restored to wild-type levels (Fig. 1A and B).

fHBP of *N. meningitidis* is important for survival in ex vivo human whole blood and human serum models of meningococcal bacteremia. *N. meningitidis* MC58, BZ83, NZ98/254, and 67/00 wild-type strains and the isogenic fHBP knockout mutants of these strains were used to investigate the role of meningococcal fHBP in ex vivo human whole blood and human serum models of meningococcal bacteremia. The human blood assay was used to assess both cellular and humoral mechanisms of killing (including the action of complement, antibody-mediated SBA, and OP, as well as killing by neutrophils, macrophages, and antimicrobial peptides), while the serum assay was used to assess killing of *N. meningitidis* mediated by the humoral immune response. The wild-type and mutant strains were incubated with human whole blood or serum for 2 hours, and samples were taken at various time points to assess survival. All of the fHBP knockout mutant strains were highly sensitive to killing by both human whole blood and human serum compared to their isogenic wild-type parent strains (1 to 2 $\log_{10}$ less survival for fHBP knockout strains than for the wild-type strains; $P \leq 0.04$) (Fig. 2 and 3). The MC58 ΔfHBP and BZ83 ΔfHBP mutant strains (high expressors) survived for less than 60 min (panels A and B), while NZ98/254 ΔfHBP (intermediate expressor) and 67/00 ΔfHBP (low expressor) survived for 120 min or longer (panels C and D). The levels of survival of mutant and wild-type strains varied between different donors and strains, most likely due to differences in complement activity, cellular killing, and levels and types of antibodies in each donor, in combination with different levels of intrinsic sensitivity to killing of the different strains. In addition, a higher level of killing was seen in blood than in serum in certain cases (donors 1 and 2 for NZ98/254 strains and

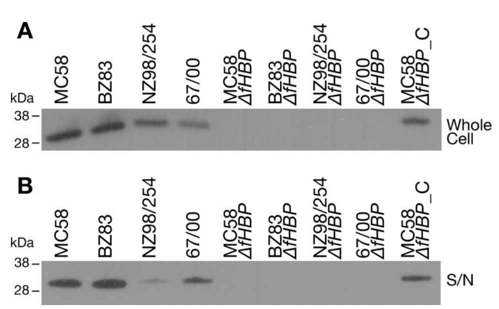

FIG. 1. fHBP expression by *N. meningitidis* strains. Western blots of whole-cell lysates (A) and culture supernatants (S/N) (B) of strains expressing high (MC58 and BZ83), intermediate (NZ98/254), and low (67/00) levels of fHBP. The fHBP isogenic knockout mutants of these strains and the complemented strain MC58 ΔfHBP_C are also shown.

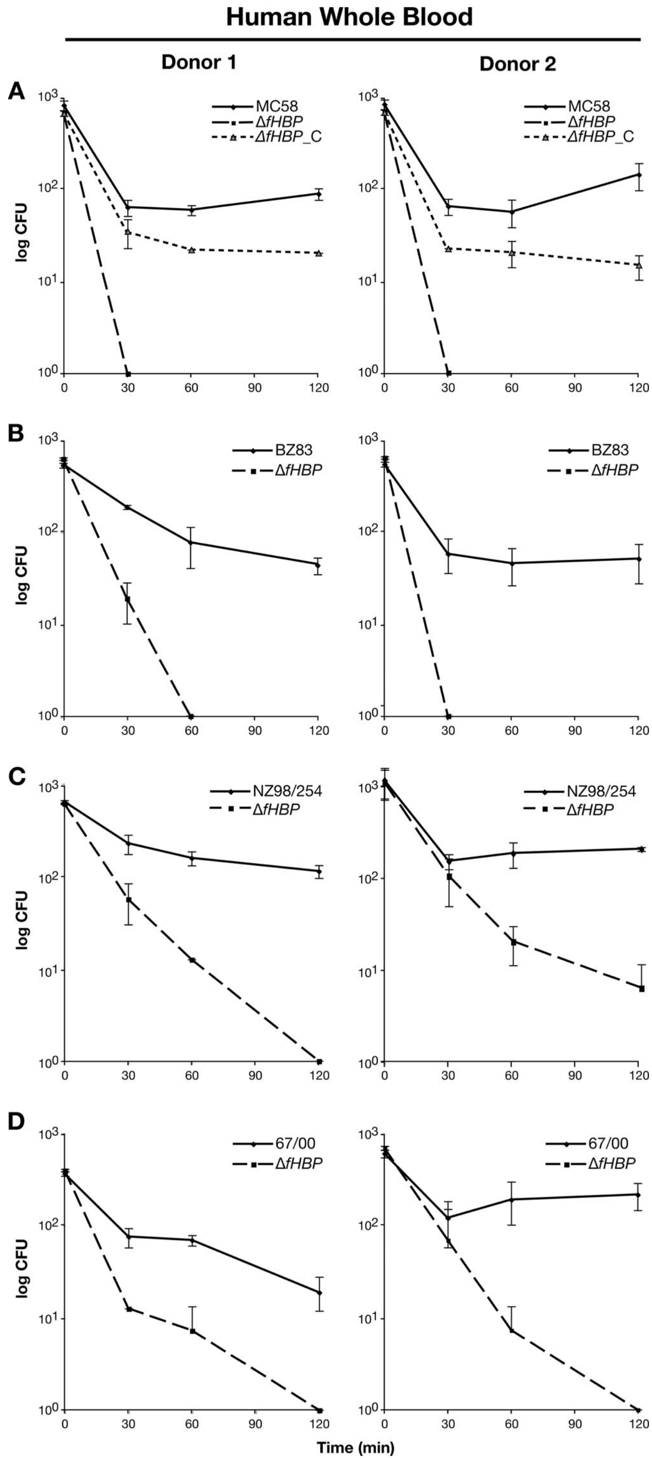

FIG. 2. Survival of wild-type and fHBP knockout strains in the ex vivo human whole blood model of meningococcal septicemia. Results show the survival of strains in human whole blood from two different donors over the course of 2 hours. (A) MC58 wild-type strain, MC58 ΔfHBP mutant strain, and MC58 ΔfHBP_C complemented strain. (B) BZ83 wild-type strain and BZ83 ΔfHBP mutant strain. (C) NZ98/254 wild-type strain and NZ98/254 ΔfHBP mutant strain. (D) 67/00 wild-type strain and 67/00 ΔfHBP mutant strain. *P* values obtained using Student's *t* test for the survival of the mutant strain or the MC58 ΔfHBP_C complemented strain with respect to the isogenic wild-type parent strain were ≤0.04 for the 120-min time point in all assays.

donor 1 for 67/00 and MC58 strains), indicating the involvement of leukocytes and OP in bactericidal activity. On the other hand, serum from donor 2 mediated more killing of BZ83 and 67/00 strains than did blood, suggesting that antibody-mediated SBA is the predominant mode of killing of these strains by this donor.

The phenotype of MC58 ΔfHBP in whole blood and serum was reversible when a single copy of the fHBP allele was provided in *trans* (MC58 ΔfHBP_C) (Fig. 2A and 3A), indicating that this phenotype is specifically mediated by fHBP. The lower level of fHBP expression seen in the MC58 ΔfHBP_C strain than in the wild-type strain (Fig. 1) is consistent with the finding that the complemented strain was not fully rescued from killing by whole blood or serum. The wild-type and fHBP knockout strains all behaved in a similar manner when they were incubated for the duration of the assay in GC broth, indicating that differences in survival were not due to intrinsic growth or survival defects (approximately threefold increase in CFU over 120 min [data not shown]). The wild-type and fHBP knockout strains also behaved in a similar manner when they were incubated in heat-inactivated serum, with no growth or killing seen over 120 min (data not shown). These results indicate that bacterial killing in serum is mediated by complement. This is consistent with the role of fHBP in providing resistance to complement-mediated killing by binding the complement inhibitor fH (25); there is no binding of fH to fHBP in the fHBP knockout strain, enabling increased killing relative to that of the wild-type strain. These results show that the expression of fHBP by *N. meningitidis* strains is important for survival in human blood and human serum, even for strains with low levels of expression.

fHBP of *N. meningitidis* is involved in resistance to killing by the antimicrobial peptide LL-37. The presence of an additional role of fHBP, i.e., protection against the antimicrobial peptide LL-37, was discovered during an investigation of the survival of wild-type and fHBP knockout strains in the presence of various antimicrobial compounds. Antimicrobial peptides are an important part of the human innate immune system which are constitutively produced by leukocytes and are also present in epithelial cells of the nasopharynx (24), the primary site of meningococcal colonization and infection. In order to determine whether fHBP is involved in protection against killing by antimicrobial peptides, in vitro survival and growth assays were performed with wild-type and fHBP knockout strains in the presence of LL-37, a short, cationic, alpha-helical peptide. MC58, BZ83, NZ98/254, and 67/00 fHBP knockout strains were more sensitive to LL-37 than their wild-type parent strains were (>1 $\log_{10}$ less survival than that of the wild type at 120 min; $P \leq 0.01$) (Fig. 4). Moreover, the MC58 wild-type phenotype was restored in the genetically complemented strain, MC58 ΔfHBP_C (Fig. 4A). Killing of *N. meningitidis* by LL-37 is dose dependent, with increasing concentrations of LL-37 from 2 to 5 μM mediating increased killing of the wild-type strains and also causing increased sensitivity of the fHBP knockout strains with respect to their wild-type parent strains (data not shown). The *N. meningitidis* strains NZ98/254 and 67/00 are intrinsically more resistant to LL-37 than MC58 and BZ/83 are (3.5 μM and 5 μM LL-37 was used in the killing assays shown in Fig. 4A and B and Fig. 4C and D, respectively). For NZ98/254 and 67/00, a <1-$\log_{10}$ decrease in

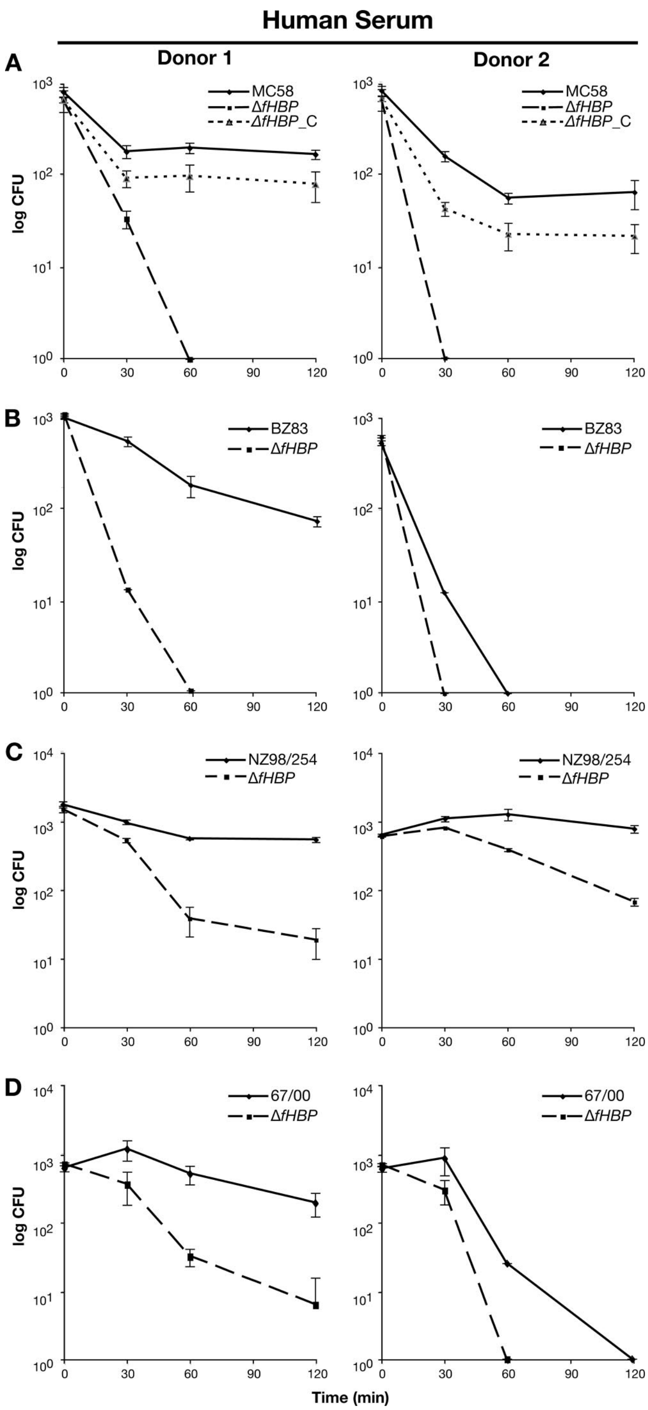

FIG. 3. Survival of wild-type and fHBP knockout strains in the ex vivo human serum model of meningococcal septicemia. Results show the survival of strains in human sera from two different donors over the course of 2 hours. (A) MC58 wild-type strain, MC58 ΔfHBP mutant strain, and MC58 ΔfHBP_C complemented strain. (B) BZ83 wild-type strain and BZ83 ΔfHBP mutant strain. (C) NZ98/254 wild-type strain and NZ98/254 ΔfHBP mutant strain. (D) 67/00 wild-type strain and 67/00 ΔfHBP mutant strain. *P* values obtained using Student's *t* test for the survival of the mutant strain with respect to the isogenic wild-type parent strain were ≤0.02 for the 120-min time point in all assays. The *P* values for survival of the MC58 ΔfHBP_C complemented strain with respect to the wild type were 0.02 and 0.07 for donors 1 and 2, respectively.

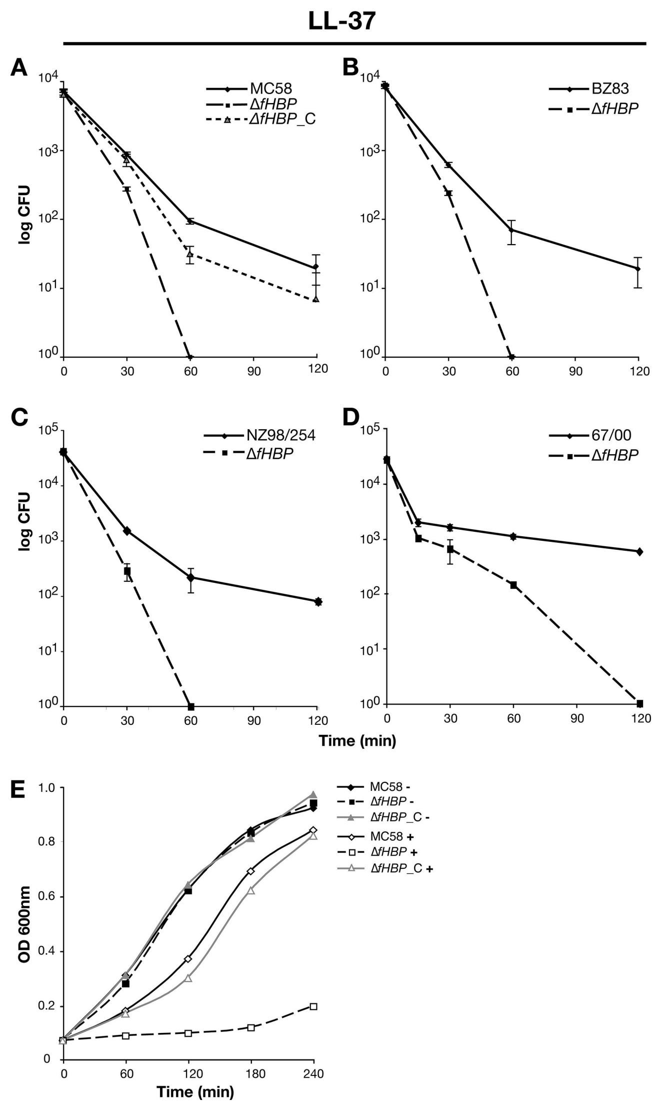

FIG. 4. Survival and growth of wild-type and fHBP knockout strains in the presence of the antimicrobial peptide LL-37. Results show the survival of strains in the presence of 3.5 μM (A and B) or 5 μM (C and D) LL-37 over the course of 2 hours. (A) MC58 wild-type strain, MC58 ΔfHBP mutant strain, and MC58 ΔfHBP_C complemented strain. (B) BZ83 wild-type strain and BZ83 ΔfHBP mutant strain. (C) NZ98/254 wild-type strain and NZ98/254 ΔfHBP mutant strain. (D) 67/00 wild-type strain and 67/00 ΔfHBP mutant strain. *P* values obtained using Student's *t* test for the survival of the mutant strain with respect to the isogenic wild-type parent strain were ≤0.01 for the 120-min time point in all assays. (E) Growth of MC58 wild-type, MC58 ΔfHBP, and MC58 ΔfHBP_C strains in the absence (−) or presence (+) of 1 μM LL-37.

CFU was seen over 2 h in the presence of 3.5 μM LL-37, compared to an almost 3-$\log_{10}$ decrease in CFU seen for MC58 and BZ/83 (data not shown). These results show that in the case of LL-37-mediated killing, fHBP is involved in protection, regardless of the fHBP expression level.

The ability of MC58, MC58 ΔfHBP, and MC58 ΔfHBP_C to grow in the presence of LL-37 at 1 μM, a concentration that only marginally affects the growth of the MC58 wild-type strain (9), was also investigated. All strains exhibited similar growth rates in the absence of peptide (with doubling times of 55 to 60 min), but the growth of MC58 ΔfHBP was significantly decreased in the presence of LL-37 (110-min doubling time) with respect to the wild-type (58-min doubling time) and complemented (68-min doubling time) strains (Fig. 4E).

In order to determine whether the fHBP knockout mutation altered the integrity of the bacterial membrane or induced a complex phenotype, leading to nonspecific sensitivity to LL-37, we performed several additional stress assays. Survival of MC58 and MC58 ΔfHBP was investigated in the presence of (i) polymyxin B, a cationic cyclic lipopeptide that is believed to have a similar mechanism of action to that of LL-37 (23, 39); (ii) oxidative stress to mimic the oxidative burst of phagocytic cells; and (iii) envelope stress, using osmotic stress and detergent stress. The MC58 ΔfHBP strain did not display increased sensitivity with respect to the wild-type strain during these in vitro killing assays (data not shown), indicating that the fHBP knockout mutation does not significantly alter the integrity of the bacterial membrane and does not cause a pleiotropic phenotype.

The bacterial mechanisms generally described for evading killing by antimicrobial peptides include active extrusion, protease digestion, sequestration, and electrostatic repulsion of the peptide (32). We investigated whether one of these mechanisms could mediate the resistance of fHBP to LL-37, and the results show that rfHBP does not have proteolytic activity toward LL-37, does not bind LL-37 in far-Western dot blot and gel filtration analyses, and does not sequester LL-37 if added to cells prior to the LL-37 killing assay (data not shown). However, MC58 and MC58 ΔfHBP had increased resistance to killing by LL-37 in the presence of 2% NaCl (disrupts electrostatic interactions) and low pH (increases the positive charge of surface-exposed proteins), indicating the presence of significant electrostatic interactions between LL-37 and the meningococcal cell surface (data not shown). The solution structure of the BC domain of fHBP revealed well-defined patches of negative and positive charges, which could mediate electrostatic interaction with or repulsion of LL-37 (26).

DISCUSSION

N. meningitidis, a commensal of the nasopharyngeal mucosa and the causative agent of life-threatening meningococcal septicemia and meningitis, is exposed to components of mucosal and systemic immunity during colonization and infection, respectively (22, 34). The ability of meningococci to colonize the mucosal epithelium as well as to survive and multiply within human blood is a key factor in the development of fulminant meningococcal disease. The importance of various factors of the immune system in preventing development of meningococcal disease is evidenced by the increased incidence and recurrence of infection and disease in people with immune disorders, including deficiencies of the terminal complement factors (C5 to C9) or complement regulator protein factors H and I (14, 27, 29, 34). *N. meningitidis* has developed many mechanisms to evade the human immune response (28), and fHBP (also known as GNA1870 and LP2086) is emerging as an important player in mediating resistance to host defenses.

fHBP is widely distributed throughout the circulating meningococcal serogroup B population and is a component of the multivalent MenB vaccine that is currently in human clinical trials as a comprehensive meningococcal serogroup B vaccine (10, 33). fHBP elicits a strong bactericidal immune response, and induced anti-fHBP antibodies have two identified modes of action, firstly by directly mediating bacteriolysis via the complement classical pathway (CP) through SBA and OP (26, 43) and secondly by blocking binding of fH, a key inhibitory regulator of the complement AP, increasing the susceptibility to killing by the complement AP (2, 25). fHBP-mediated interactions with the host immune response and the mechanisms by which vaccine-induced anti-fHBP antibodies contribute to protection against meningococcal infection are outlined in Fig. 5.

Here we show that fHBP of *N. meningitidis* is important for survival in ex vivo models of bacteremia using normal human blood and serum, regardless of the level of its expression by different strains. Recent findings indicate that unlike fHBP of high expressors, fHBP of low expressors is not required for survival in nonimmune human blood (43). The differences between the results of these two studies are likely due to donor selection. In comparison to nonimmune blood, where wild-type strains grow or maintain the initial bacterial counts during the experiment, the assay reported herein uses human blood and serum, which mediate some killing of the wild-type strain (0.5- to 1-$\log_{10}$ decrease in CFU over 2 h), most likely due to background levels of antibodies to *N. meningitidis* or of cross-reactive antibodies within the blood of the donors. Using the same nonimmune donor serum from the previous study, we were able to replicate the finding that NZ98/254 ΔfHBP did not have greatly reduced survival relative to that of the wild-type strain (43; data not shown).

The findings from this study indicate the importance of a third mode of action by which anti-fHBP antibodies induce a bactericidal response (Fig. 5B). fH, by controlling the key steps in the complement AP amplification cycle, also dampens activation that has been initiated via the complement CP or the lectin pathway (13, 15). Hence, antibody-mediated blocking of fH binding to fHBP enables amplification of the complement CP and enhances CP-mediated killing in the presence of other antibodies. Therefore, even expressed at low levels, fHBP likely plays a role in resistance to *N. meningitidis* by dampening activation of the complement CP that occurs in the presence of antimeningococcal antibodies in normal blood. Conversely, in the absence of fH binding (fHBP knockout strains or blocking of binding by antibodies) (2, 25), AP complement-mediated killing of *N. meningitidis* is derepressed, and the effect of the complement CP may be amplified. These findings are particularly interesting in the context of the general population or a population vaccinated with a multivalent vaccine containing fHBP, both of which would be expected to have an array of antimeningococcal antibodies. Colonization by *N. meningitidis*, *Neisseria lactamica*, or certain enteric bacteria has been reported to induce an immunologic response such that by young adulthood the majority of people have measurable levels of antibody to the pathogenic meningococcal serogroups (8, 12, 35). In addition, more than 50% of newborn infants have maternal bactericidal antibodies to the major meningococcal serogroups, whose prevalence wanes after birth and is lowest from 6 to 24 months of age (11). This correlates with the highest rate of serogroup B meningococcal disease occurring in infants under the age of 1 year (5). This age group is the main

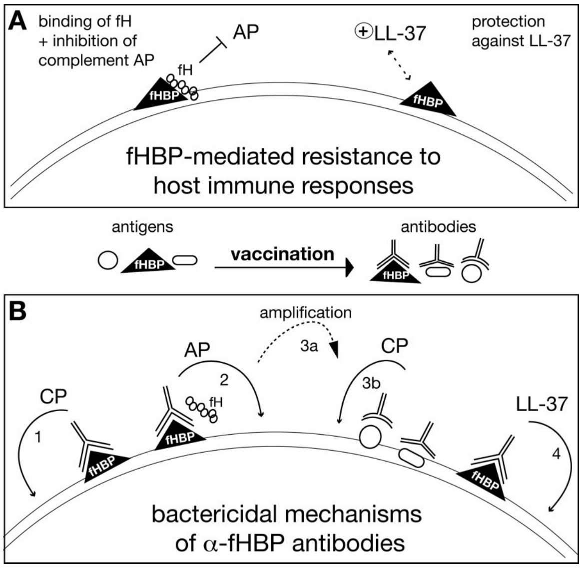

FIG. 5. Model of fHBP-mediated interactions with the host immune response (A) and mechanisms by which vaccine-induced anti-fHBP antibodies contribute to protection against meningococcal infection (B). (A) Binding of fH by fHBP leads to inhibition of the complement AP. fHBP also provides protection against killing by the cationic antimicrobial peptide LL-37, by an unknown mechanism. (B) Anti-fHBP antibodies induce bactericidal activity via several mechanisms, as follows. (1) Direct activation of the complement CP, leading to complement-dependent SBA and OP; (2) prevention of inhibition of the AP by blocking of fH binding, leading to AP-mediated killing; (3) amplification of the CP (3a) in the absence of AP inhibition, leading to increased bactericidal activity of other antimeningococcal antibodies (3b); and (4) disruption of interactions between LL-37 and fHBP may lead to increased destabilization of the bacterial cell by LL-37.

target of a comprehensive serogroup B vaccine. The MenB vaccine was recently administered to 150 healthy UK infants concomitantly with routine immunizations at 2, 4, and 6 months of age, with a booster dose at 12 months (30).

From studies using MAbs, it has been suggested that multiple sites on the surface of fHBP may interact with fH (2). Hence, vaccine-induced polyclonal antibodies raised to fHBP variant 1, even if not bactericidal or directly protective against strains carrying other fHBP variants, may still interfere with fH binding, thus enabling amplification of the immune response induced by the other antigens of a multicomponent vaccine.

We have identified an additional role of fHBP, i.e., protection against killing by the cationic antimicrobial peptide LL-37, in high, intermediate, and low expressors (Fig. 5A). Since LL-37 is produced by cells that interact with *N. meningitidis* during infection, including the nasopharyngeal epithelia and phagocytic cells of the blood, it may be involved in innate host defenses against meningococcal disease. The in vivo role of LL-37 in antimicrobial host defense has been demonstrated for several pathogens (reviewed in reference 20). The direct mechanisms of LL-37-dependent microbial killing are not completely understood, but LL-37 is believed to act through a blanket effect by interaction of its positive charges with the negatively charged bacterial surface, causing destabilization and permeabilization of the membrane. In response, bacteria have evolved several mechanisms for evading killing by antimicrobial peptides, including (i) active extrusion via an export complex, (ii) protease digestion of the peptide, (iii) sequestration by secreted proteins, and (iv) alteration of the surface charge to prevent electrostatic interactions (32). While the mechanism of fHBP-mediated resistance to antimicrobial peptides remains elusive, our data suggest that the sensitivity of fHBP-deficient strains is not attributable to decreased outer membrane stability, nor is fHBP involved in protease activity, efflux, or sequestration of LL-37. We have not ruled out the possibility of an indirect effect of fHBP in resistance to LL-37, but it is unlikely that fHBP has a secondary effect on the MtrCDE efflux pump, since wild-type and mutant strains displayed the same sensitivity to polymyxin B, unlike meningococcal MtrCDE mutant strains, which have increased sensitivity to both LL-37 and polymyxin B (42). fHBP-mediated protection against LL-37 is most likely mediated by electrostatic interactions which prevent interaction of LL-37 with the cell surface. The solution structure of the immunodominant BC domain of fHBP revealed an extensive hydrophobic region on one side of the molecule that may interact with the outer membrane or other molecules, while the other face has a well-defined negative patch and diverse patches of positive charge (26), which could mediate electrostatic interaction with LL-37, preventing contact with the cell membrane.

LL-37 may contribute to the sensitivity of the fHBP knockout strains seen in the ex vivo blood and serum assays described above. Since fHBP binds specifically to human fH, but not rat or mouse fH (38), the fact that nonbactericidal antibodies raised against fHBP can provide passive protection in an infant rat model (16) supports the presence of an additional role of fHBP.

fHBP is an attractive vaccine antigen due to its complex interactions with the immune system and the fact that anti-fHBP antibodies have multiple modes of inducing a bactericidal response. In addition, the functional role of fHBP in survival of *N. meningitidis* in ex vivo (human serum and whole blood) and in vitro (antimicrobial peptide LL-37) infection models may decrease the likelihood of vaccine-induced selection of mutants lacking fHBP.

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